Binding of factor H to tubular epithelial cells limits interstitial complement activation in ischemic injury

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Factor H is a regulator of the alternative pathway of complement, and genetic studies have shown that patients with mutations in factor H are at increased risk for several types of renal disease. Pathogenic activation of the alternative pathway in acquired diseases, such as ischemic acute kidney injury, suggests that native factor H has a limited capacity to control the alternative pathway in the kidney. Here we found that an absolute deficiency of factor H produced by gene deletion prevented complement activation on tubulointerstitial cells after ischemia/reperfusion (I/R) injury, likely because alternative pathway proteins were consumed in the fluid phase. In contrast, when fluid-phase regulation by factor H was maintained while the interaction of factor H with cell surfaces was blocked by a recombinant inhibitor protein, complement activation after renal I/R increased. Finally, a recombinant form of factor H, specifically targeted to sites of C3 deposition, reduced complement activation in the tubulointerstitium after ischemic injury. Thus, although factor H does not fully prevent activation of the alternative pathway of complement on ischemic tubules, its interaction with the tubule epithelial cell surface is critical for limiting complement activation and attenuating renal injury after ischemia.

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The alternative pathway of complement (CAP) is a phylogenetically ancient arm of the innate immune system that eliminates invasive pathogens and facilitates the removal of injured host cells.[1] Inadequately controlled CAP activation has also been implicated in the pathogenesis of a diverse group of diseases including renal ischemia/reperfusion (I/R) injury, atypical hemolytic uremic syndrome (aHUS), dense deposit disease, age-related macular degeneration, and asthma.[2] The important role of this innate immune system in such a diverse group of diseases suggests that CAP activation may be a general response to many types of tissue injury. Furthermore, the important role for CAP activation in many types of renal disease indicates that the kidney may be particularly susceptible to injury by this component of the immune system.

The CAP is continually autoactivated in the fluid phase through a process called 'tickover'. Tickover results in the spontaneous generation of C3b, which can covalently bind to nearby biologic surfaces. Tissue-bound C3b can then catalyze further complement activation through the CAP, unless the reaction is effectively controlled by complement regulatory proteins (CRPs). Tickover permits the rapid elimination of pathogens that do not bear CRPs, and expression of CRPs can be regarded as a recognition mechanism by which the CAP distinguishes healthy host cells from injured cells and invasive pathogens.[3] However, continual initiation of the CAP renders host cells critically dependent upon control of CAP activation by CRPs to prevent spontaneous complement-mediated injury. Recent studies have demonstrated that mutations in the CRPs (particularly factor H) are strong risk factors for aHUS[4] and dense deposit disease.[5] It is noteworthy that these system-wide defects in CAP regulation frequently cause disease that is localized to the kidney, and the reason for this is currently unknown.

Factor H is an ~155-kDa glycoprotein that circulates in high concentrations (0.3–0.5 mg/ml) and regulates CAP activation in the fluid phase as well as on tissue surfaces.[6]

CAP inhibition by factor H on a given tissue requires that factor H interacts properly with the tissue surface. Several regions within the factor H protein bind to anionic surfaces, such as membranes rich in heparan sulfate or sialic acid, as well as to tissues bearing C3b or C3d.[7,8] The polymorphisms and mutations associated with increased risk for developing age-related macular degeneration and aHUS most frequently involve binding regions of factor H and not the complement regulatory domain.[4,9] Activation of the CAP within a given tissue, therefore, is strongly influenced by the affinity of factor H for that tissue surface. The tropism of these diseases for specific organs also suggests that these particular tissues or cell types are dependent upon factor H for CAP regulation, and that the relative affinity of factor H for tissue-specific surfaces is an important factor in the development of disease.

The CAP also mediates tissue injury in the presence of fully functional factor H. Ischemic acute kidney injury in wild-type rodents[10,11] and in humans[12] is associated with pathologic activation of the CAP on the basolateral surface of injured tubular cells. We have found that complement receptor 1-related gene/protein y (Crry, a rodent analog of human membrane cofactor protein (CD46) and CR1) is the only cell surface CRP expressed by proximal tubular epithelial cells (TECs) in mice, and that IR alters the basolateral localization of this protein.[13] Uncontrolled activation of the CAP in the setting of reduced surface Crry indicates that circulating factor H, despite its high protein levels, has a limited ability to protect the surface of hypoxic TECs, leaving the ischemic kidney susceptible to pathologic CAP activation. Given the role of CAP activation in the pathogenesis of such a wide variety of acquired diseases, it is important to understand the mechanisms by which endogenous complement regulation can be overwhelmed or subverted. Because complement activation in ischemic acute kidney injury occurs almost exclusively via the CAP, it is an informative model in which to study these mechanisms.

We hypothesized that circulating factor H fails to prevent CAP activation on hypoxic tubules because of insufficient affinity for the TECs. To distinguish the role of factor H in fluid-phase and cell surface CAP inhibition, we have used several tools, including mice with targeted deletion of the factor H gene ($fH^{-/-}$ mice that lack fluid-phase and surface CAP regulation by factor H) and a recombinant protein that competes with one of the binding regions in factor H to block surface CAP regulation by the protein. We have also employed a recombinant protein that specifically targets factor H to sites of iC3b/C3d deposition in order to examine whether inhibition of CAP by factor H on tissues can be improved by increasing the affinity of factor H for the cell surface. Using these tools we have performed *in vivo* and *in vitro* studies to explore the interaction of factor H with the TEC surface. The results of these studies reveal a complex role for native factor H in regulating the CAP in acquired inflammatory diseases and also suggest novel methods for ameliorating tissue injury.

RESULTS

Targeted deletion of the factor H gene reduces tubulointerstitial C3 deposition after renal I/R

Mice genetically deficient in factor H develop complement-mediated glomerular injury as they age.[14] Complement activation in these mice occurs in the fluid phase because of the lack of regulation by factor H, and levels of circulating intact C3 are low in these mice (Figure 1a, see Rose *et al.*[15]). C3a levels in the plasma of these mice are elevated, however, reflecting the high rate of fluid-phase complement activation (Figure 1b). We subjected $fH^{-/-}$ mice to renal I/R. The serum urea nitrogen (SUN) and creatinine values in the $fH^{-/-}$ mice showed a trend toward being lower than in the wild-type controls (Figure 1c and d), suggesting that the absence of circulating factor H may be beneficial. The failure of this protection to reach significance may be because of the generation of proinflammatory factors in the plasma of $fH^{-/-}$ mice, which counteracts the reduction in tubulointerstitial complement activation. Tubular injury was seen in the outer medullas of $fH^{-/-}$ mice and wild-type mice subjected to I/R (Figure 1e and f). Immunofluorescence microscopy demonstrated that there was little tubular deposition of C3 in the $fH^{-/-}$ mice, indicating that uncontrolled fluid-phase activation of C3 in these mice does not leave sufficient intact C3 to support complement activation on the TEC surface after I/R. Wild-type mice subjected to renal I/R demonstrated widespread C3 along the tubular basement membrane of injured tubules (Figure 1m and n). It has been determined that the glomerular deposits of C3 fragments in $fH^{-/-}$ mice require the presence of factor I.[15] These results indicate that it is the iC3b fragment of C3 that binds to the glomerular basement membrane of these mice. It is not clear, however, why this is the particular site of iC3b deposition.

Factor H limits complement-mediated injury after renal I/R in wild-type mice

In order to distinguish the function of factor H on cell surfaces from its fluid-phase function, we generated a murine recombinant protein (rH19-20) that competitively blocks binding of the carboxy terminus of factor H to cell surfaces, similar to the human homolog that has previously been described.[16,17] We confirmed *in vitro* that the rH19-20 competes with intact factor H in a dose-dependent manner for binding to C3b-coated host-like cell surfaces (sheep erythrocytes; Figure 2a). These cells are normally resistant to complement-mediated lysis, but we found that at concentrations of rH19-20 that inhibit factor H binding, rH19-20 permits CAP-mediated lysis of these cells in a dose-dependent manner (Figure 2b).

The CAP is activated along the basolateral aspect of tubules in the outer medulla after renal I/R.[13,18] To confirm that we could achieve comparable levels *in vivo*, we injected wild-type mice with 50 μg of biotinylated rH19-20 and measured serum levels of the protein (Figure 3a). At 15 min after injection of the rH19-20, the serum levels were 0.46 ± 0.06 μmol/l. To test whether circulating factor H is

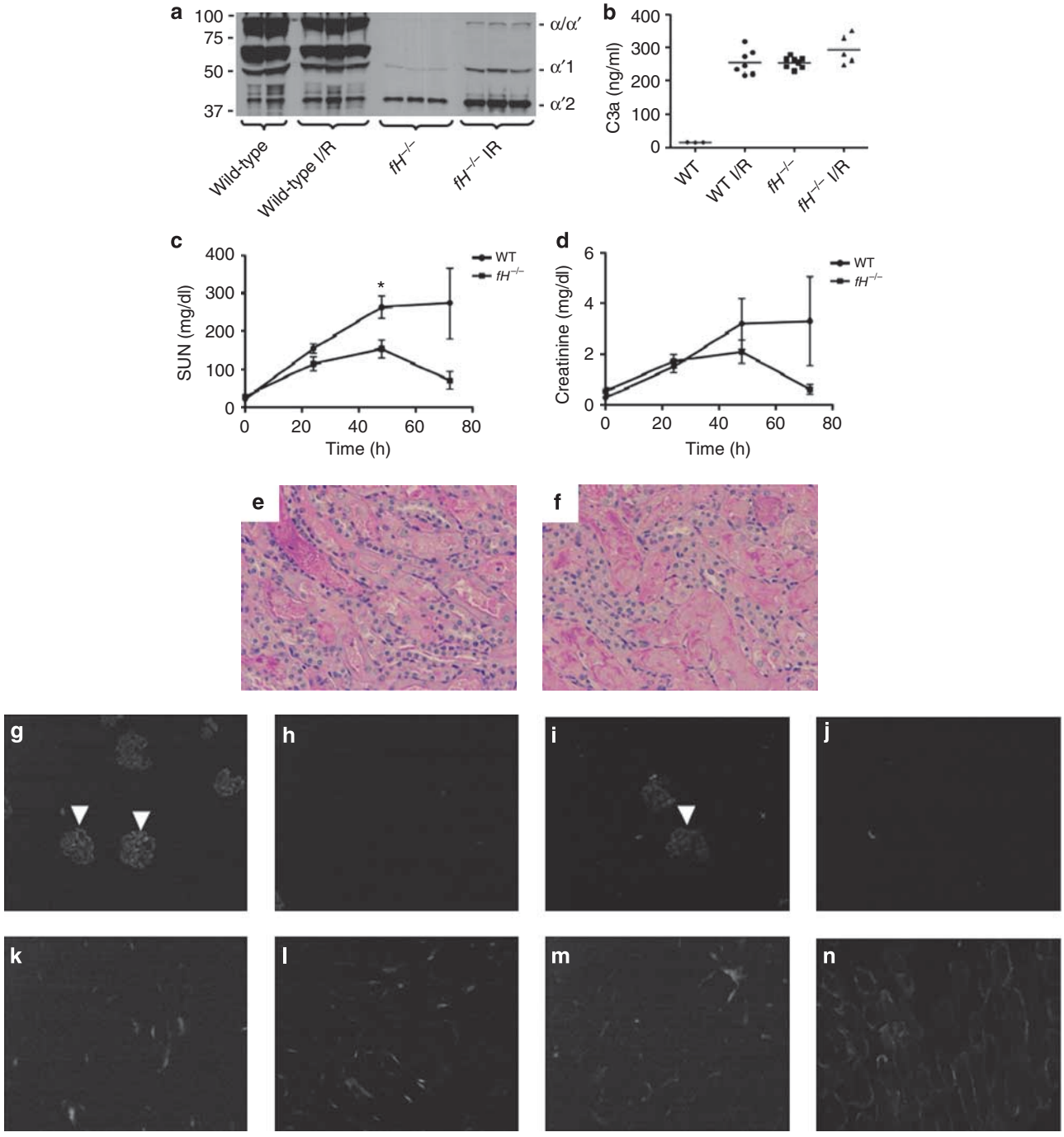

Figure 1 | Complement activation is reduced in the tubulointerstitium of mice lacking factor H. $fH^{-/-}$ mice and wild-type (WT) controls were subjected to renal ischemia/reperfusion (I/R). (**a**) Western blot analysis for C3 demonstrated that most of the circulating C3 in $fH^{-/-}$ mice is cleaved and is detected as α′1 or α′2 fragments. (**b**) Plasma C3a levels were measured by enzyme-linked immunosorbent assay. Levels in $fH^{-/-}$ mice were higher than in WT mice, and were comparable with those seen in WT mice subjected to renal I/R. (**c**) Serum urea nitrogen (SUN) levels and (**d**) creatinine levels were lower in the $fH^{-/-}$ mice, although this only achieved statistical significance for SUNs and only at 48 h of reperfusion (*$P < 0.05$). Sham-treated animals are presented as 0 h. Tubular necrosis was seen in the kidneys of (**e**) WT and (**f**) $fH^{-/-}$ mice. Immunofluorescence microscopy for C3b in the kidney of a sham-treated $fH^{-/-}$ mouse demonstrated (**g**) heavy deposition of C3b in the glomeruli, but (**h**) no C3b in the tubulointerstitium of the outer medulla. In $fH^{-/-}$ mice subjected to renal I/R, C3b deposition was still not seen along the tubules of the (**i**) cortex or (**j**) outer medulla. In WT mice, C3b was not seen in the glomeruli of (**k**) sham-treated or (**m**) postischemic mice, but was seen in the tubules of the cortex and outer medulla (**l** and **n** are sham and postischemia, respectively). $n = 5$ for each group. Original magnification × 400 for **b** and **c**, staining is with periodic acid-Schiff. Original magnification × 200 for **d**–**k**. Glomeruli are indicated with arrowheads.

functionally important in limiting complement activation at this location, we subjected wild-type mice to renal I/R, and then injected them with rH19-20. When biotinylated rH19-20 was used, the protein could be detected on the injured tubules in the outer medulla (Figure 3b). The pattern of C3 deposition within the kidney was extensive in both control animals (Figure 3d) and in mice treated with the rH19-20 (Figure 3e). Mice injected with rH19-20 demonstrated greater systemic C3a than controls (Figure 3g), indicating a greater overall degree of complement activation. Semiquantitative assessment of tubular C3 did not show a significant change in mice treated with the rH19-20 (Figure 3f), possibly because it is difficult to detect the increase of tubulointerstitial complement activation above the already extensive activation seen in wild-type animals.

SUN levels were significantly higher in mice treated with rH19-20 (Figure 4a), indicating significant functional impairment in mice treated with rH19-20. Creatinine values were not significantly higher in mice that received rH19-20 (Figure 4b), although most of the mice that received rH19-20 died before measurements at later time points could be made (5 mice were treated: 3 died before 48 h of reperfusion and all died before 72 h of reperfusion). Renal I/R typically causes injury of the tubules in the outer medulla. Injection of sham-treated mice had no detectable effect on morphology in the cortex or the outer medulla (Figure 4c). Control mice subjected to renal I/R showed tubular injury in the outer medulla (Figure 4d). In mice treated with rH19-20 after I/R, however, the injured tubules extended into the cortex (Figure 4e). These results indicate that although the CAP is activated on the tubules after renal I/R, the overall degree of CAP activation is limited by factor H.

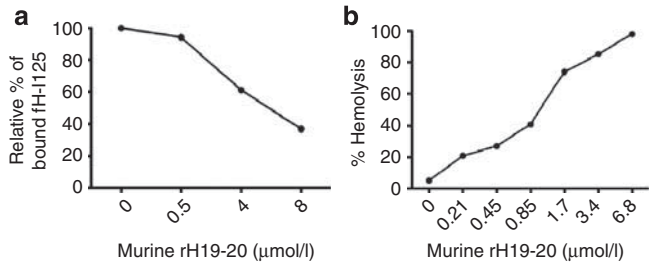

Figure 2 | Murine rH19-20 (rH19-20) blocks complement inhibition by factor H on cell surfaces. (**a**) Cells bearing C3b were incubated with 20 ng of radioiodinated full-length factor H and the stated concentration of unlabeled rH19-20. After 20 min at 22 °C, the bound and free radiolabels were separated by centrifugation of cells through 20% sucrose. The results are graphed relative to the binding observed with labeled factor H in the absence of rH19-20. (**b**) Sheep erythrocytes were incubated in 40% normal mouse serum in the presence of rH19-20 at the stated concentrations for 20 min at 37 °C. The assays contained 5 mmol/l MgEGTA to inhibit classical and lectin pathway activation and restrict activation to the calcium-independent alternative pathway of complement. Lysis was subsequently measured by hemoglobin release (A412) after centrifugation to remove unlysed cells.

Native factor H controls complement activation on the surface of TECs *in vitro*

We have previously found that there is spontaneous complement deposition on TECs exposed to 10% homologous

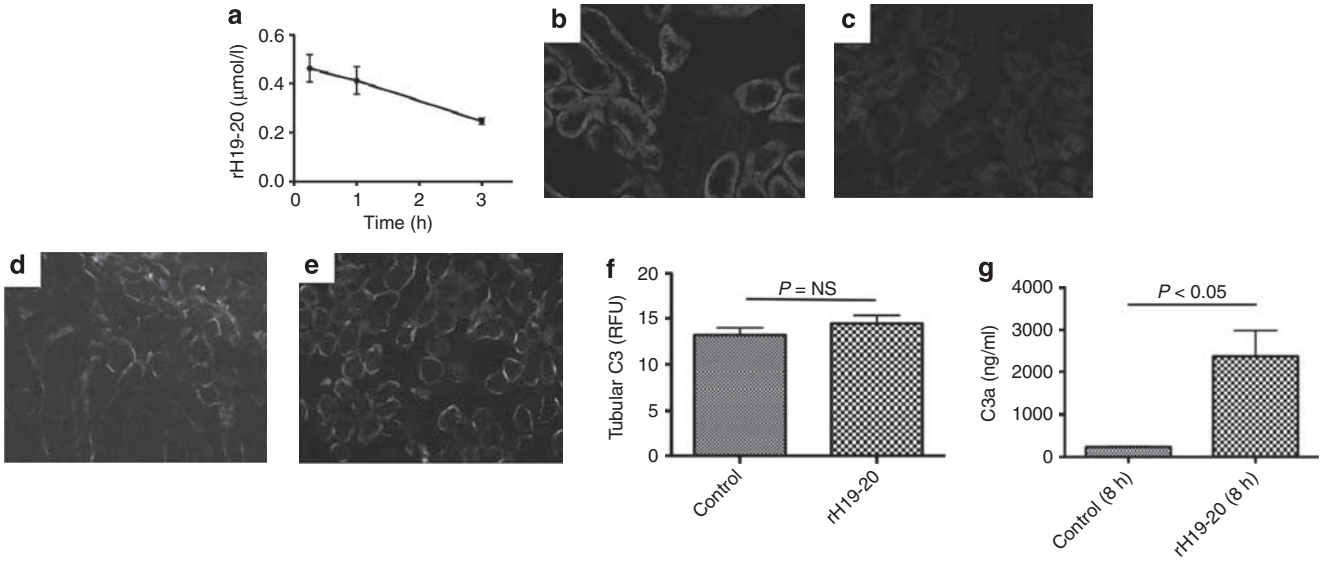

Figure 3 | Surface-bound factor H limits complement activation after renal ischemia/reperfusion (I/R). (**a**) Mice were injected with 50 μg of rH19-20, and serum levels were measured by enzyme-linked immunosorbent assay ($n = 4$). Wild-type mice were subjected to renal I/R and were treated with biotinylated rH19-20 or with vehicle. Immunofluorescence microscopy demonstrated the protein along the basolateral aspect of injured tubules (**b**). No protein was detected in vehicle-treated controls (**c**). Widespread deposition of C3 was seen in the tubulointerstitium of (**d**) vehicle- and (**e**) rH19-20-treated animals by immunofluorescence microscopy. (**f**) Semiquantitative scoring of tubulointerstitial C3 was not significantly greater in mice injected with rH19-20 than in mice injected with vehicle. (**g**) Plasma levels of C3a were higher in the mice receiving rH19-20 than in the control animals. $n = 6$ for each group. Original magnification × 200 for **b**–**e**.

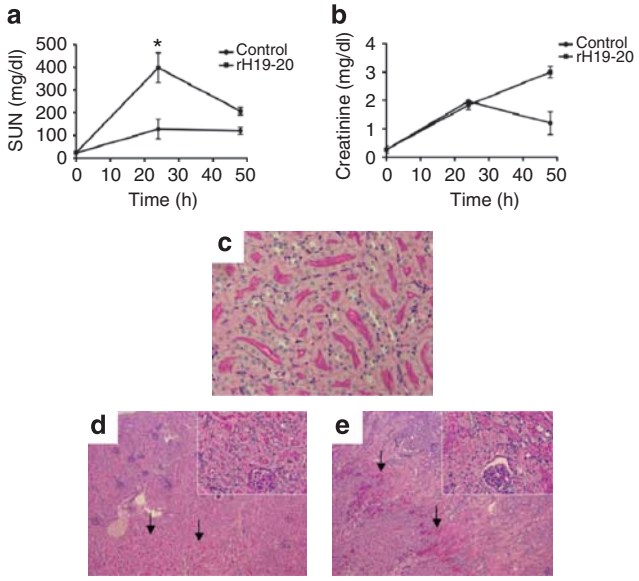

Figure 4 | Surface-bound factor H limits complement activation after renal ischemia/reperfusion (I/R). (**a**) Serum urea nitrogen (SUN) levels were higher in the mice treated with 50 μg rH19-20 than in vehicle-treated controls at the 24 h time point (*$P<0.05$). (**b**) Creatinine levels were not significantly different between rh19-20-treated and control mice. (**c**) Tubular injury was not seen in sham-treated mice that received the rH19-20. Tubular necrosis was seen in the outer medullas of (**d**) control and (**e**) rH19-20-treated mice (denoted with arrows), but the tubular damage in the rH19-20 mice was more extensive and was seen in the cortex (inset). Sham-treated animals are presented as 0 h in **a** and **b**. Original magnification $\times 200$ for **c** and $\times 100$ for **c** and **e** ($\times 400$ for inset view), staining is with periodic acid-Schiff. $n=6$ at 24 h, $n=2$ at 48 h.

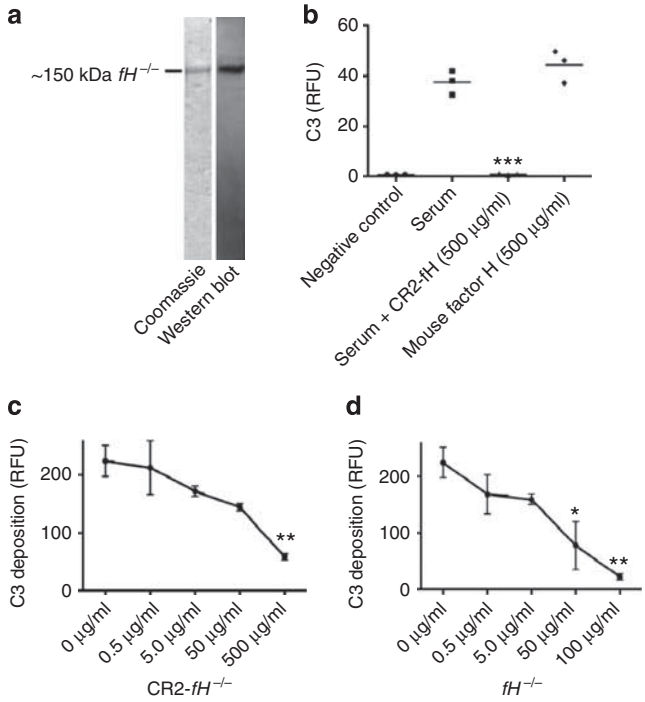

Figure 5 | Factor H and CR2-fH inhibit complement activation on tubular epithelial cells (TECs). (**a**) Factor H was purified from the serum of wild-type mice. Its purity and identity were confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie and western blot analysis. (**b**) The ability of both factor H and CR2-fH to inhibit complement activation on zymosan particles was tested. High concentrations of factor H did not reduce the amount of C3 deposited on zymosan particles by 10% mouse serum when assessed by flow cytometry, but CR2-fH did inhibit complement activation on the surface of the particles. (**c**) Increasing concentrations of CR2-fH prevented deposition of C3 on the surface of TECs exposed to 10% normal mouse serum when assessed by flow cytometry. (**d**) Increasing concentrations of factor H also prevented deposition of C3 on the surface of TECs exposed to normal mouse serum. The results are the average of three independent experiments. $n=3$ for panels **b**–**d**. ***$P<0.001$ versus serum alone. **$P<0.01$ versus no CR2-fH or factor H. *$P<0.05$ versus no factor H.

serum in spite of the factor H present within the serum. The nature of tissue surfaces influences the efficacy of factor H at regulating CAP activation on that surface.[8] Therefore, we wanted to test whether complement activation on TECs is because of the inability of factor H to regulate the CAP on the surface of these cells. To do this, we purified factor H from the plasma of wild-type mice (Figure 5a). Zymosan particles are a known CAP activator, in part because factor H does not interact well with the particle surface. The addition of exogenous factor H to serum did not attenuate complement activation on the surface of zymosan particles, demonstrating poor interaction of factor H with the zymosan surface (Figure 5b). However, a recombinant protein that targets the inhibitory region of mouse factor H specifically to sites of C3b/iC3b/C3d deposition did fully prevent complement activation on the zymosan particles (Figure 5b). This protein comprises C3d-binding region of complement receptor 2 linked to the first five short consensus repeats (the complement inhibitory region) of factor H (referred to as CR2-fH;[19]), a strategy that has previously been utilized for the targeting of complement inhibitors to sites of complement activation.[20,21] Functional superiority of the CR2-fH compared with endogenous factor H at preventing complement activation on a given surface can be attributed to differential binding of the protein to the specific surface in question as the two proteins contain the same complement regulatory moiety.

We next tested the ability of purified mouse factor H and CR2-fH to prevent complement activation on the surface of TECs. Increasing concentrations of CR2-fH led to full inhibition of complement activation on the cell surface (Figure 5c). Increasing concentrations of factor H also effectively prevented complement activation on the TEC surface. Given that factor H was also present in the serum added to the cells (~ 500 μg/ml), the final concentrations of factor H in these experiments were superphysiologic. Nevertheless, these results demonstrate that at a high enough concentration, factor H is capable of suppressing CAP activation on the TEC surface.

Targeted factor H prevents complement activation after renal I/R

We subjected mice to renal I/R and injected them with 250 or 500 μg of CR2-targeted factor H after 15 min of reperfusion. When CR2-fH was injected into mice that had already been subjected to ischemia and 16 h of reperfusion (to allow tubular complement deposition), the CR2-fH could be detected in the tubulointerstitium (Figure 6a). When injected shortly after reperfusion, the CR2-fH reduced tubulointerstitial C3 deposition. It did not reduce glomerular C3 deposition, although this is not a primary site of complement activation in this model (Figure 6f and h). We found that the CR2-targeted factor H ameliorated renal injury after 24 h of reperfusion as assessed by SUN and creatinine (Figure 7a and b). Lower SUN and creatinine values were seen in CR2-fH-treated mice out to 72 h of reperfusion, but the values were not significantly different than control-treated mice at these time points. Tubular injury was still seen in the CR2-fH-treated mice (Figure 7c). Targeted agents that can deliver factor H to the tubulointerstitium can, therefore, be used to prevent CAP activation in the damaged kidney. Three mice were treated with high-dose CR2-fH (500 μg). The high-dose CR2-fH did not confer superior protection to that achieved with 250 μg. The SUN level was 72 ± 5 mg/dl and the creatinine level was 1.6 ± 0. Tubular injury was still seen in kidneys of the high-dose group (Figure 7e).

DISCUSSION

The experiments in this study highlight the dual role of factor H in regulating CAP activation. Circulating factor H prevents consumption of C3 in the fluid phase, thus maintaining adequate levels of C3 to support complement activation on cell surfaces. Consequently, little tubulointerstitial complement activation was observed in $fH^{-/-}$ mice subjected to renal I/R. On the other hand, the interaction of the carboxy terminus of factor H with host cells limits CAP-mediated inflammation on tissue surfaces. When we blocked this interaction in wild-type mice by treating them with rH19-20, the mice displayed greater tubulointerstitial complement activation and more severe injury. Therefore, although

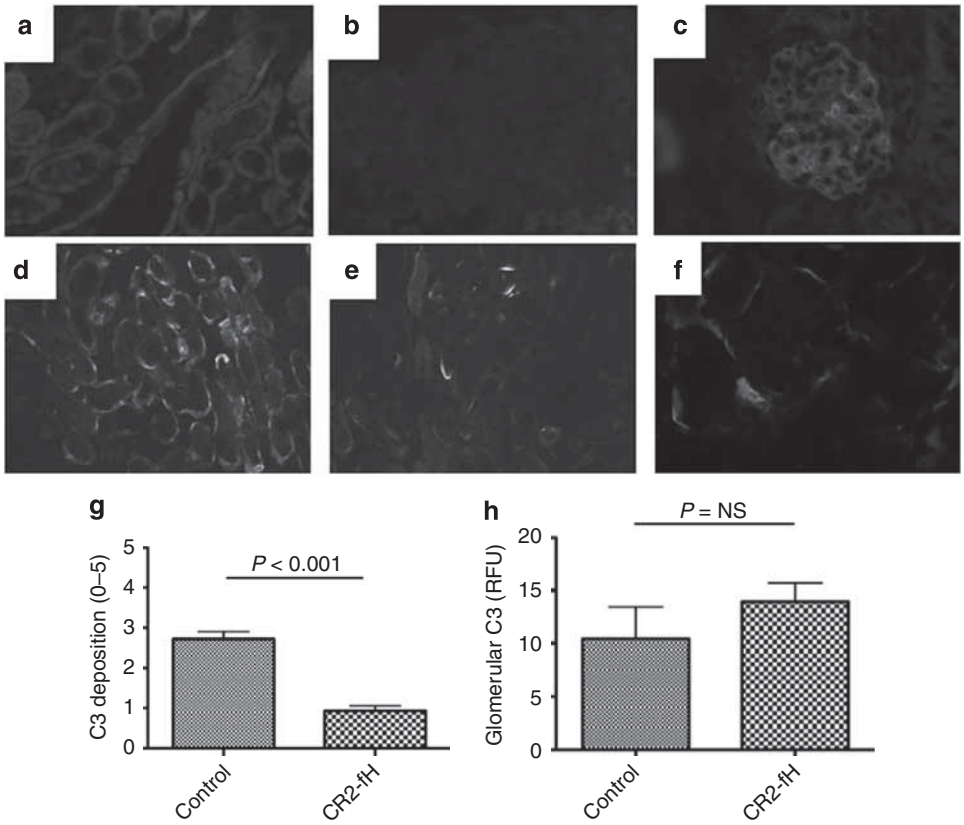

Figure 6 | CR2-fH prevents complement activation in the kidney after ischemia/reperfusion (I/R). Wild-type mice were subjected to renal I/R and were then treated with 250 μg of CR2-fH. (**a**) In mice that received the CR2-fH after 16 h of reperfusion, the protein was detected within the tubulointerstitium. (**b**) No protein was detected in vehicle-treated controls. (**c**) $fH^{-/-}$ mice were injected with CR2-fH, and the protein was detected in the glomeruli (the site of C3 deposition), not in the tubulointerstitium. (**d**) Vehicle-treated animals have widespread tubulointerstitial C3 deposition after 24 h of reperfusion. (**e**) When CR2-fH was injected within 15 min of reperfusion, the extent of C3 deposition was significantly reduced. (**f**) Little glomerular C3 is detected in mice subjected to I/R. (**g**) Semiquantitative assessment of C3 deposition demonstrated that CR2-fH reduced the tubulointerstitial complement activation ($n = 8$–10). (**h**) Semiquantitative assessment of the glomerular C3 did not demonstrate any effect of treatment with CR2-fH on glomerular complement activation ($n = 5$). Original magnification × 200 for **a**, **b**, **d**, and **e**; original magnification × 400 for **c** and **f**.

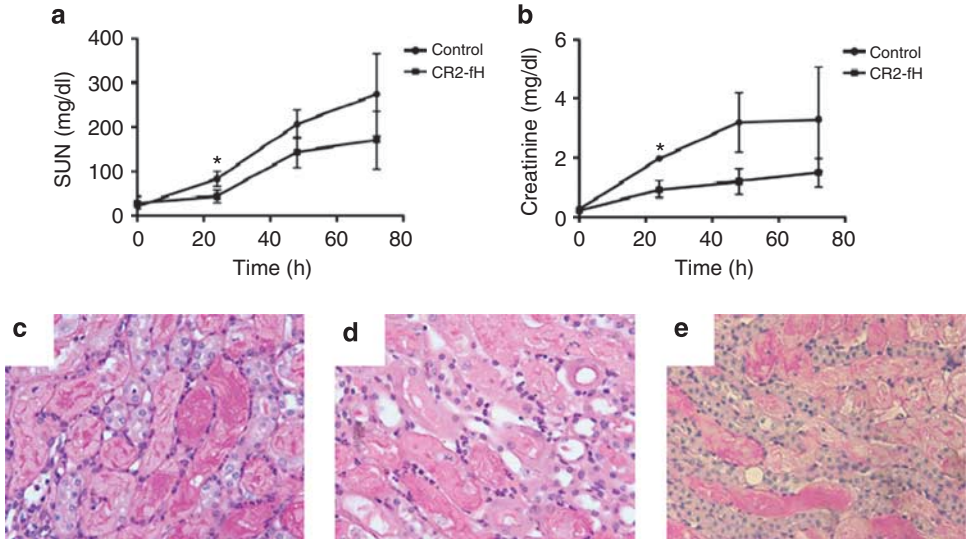

Figure 7 | CR2-fH attenuates renal injury after ischemia/reperfusion (I/R). Wild-type mice were subjected to renal I/R and were then treated with 250 μg of CR2-fH. (**a**) Serum urea nitrogen (SUN) levels and (**b**) creatinine levels were significantly lower in the mice that received CR2-fH than in vehicle-treated controls after 24 h of reperfusion. In spite of the functional protection provided by CR2-fH, tubular injury was still seen in both (**c**) vehicle-treated and (**d**) CR2-fH-treated animals. (**e**) Tubular injury was still seen in mice treated with a higher dose of CR2-fH (500 μg). $n = 11$ for vehicle control and $n = 10$ for CR2-fH treatment at the 24 h time point. $n = 4$–6 for the later time points. Sham-treated animals are presented as 0 h. Original magnification $\times 200$ for **c**–**e**, staining is with periodic acid-Schiff.

factor H does not fully prevent CAP activation on ischemic tubules, interaction of factor H with the TEC surface is critical for limiting complement activation after renal I/R.

The efficacy of factor H at regulating CAP activation on a given surface is a function of its affinity with that surface.[8] High concentrations of factor H did not prevent CAP activation on zymosan particles (an 'unreceptive' surface), but did prevent CAP activation on TECs in culture. CR2-fH, which specifically targets the complement regulatory region of factor H to sites of complement activation, effectively prevented complement activation on both unreceptive (zymosan) and receptive (TEC) surfaces. CR2-fH also effectively prevented CAP activation in the renal tubulointerstitium after I/R. This strategy can therefore prevent CAP activation on tissues where endogenous factor H does not fully prevent CAP activation because of insufficient quantity or affinity for the surface, effectively overcoming the limitations of the native protein.

Mutations and polymorphisms in the tissue-binding regions of factor H have been linked to several diseases, including aHUS and age-related macular degeneration. Conversely, CAP-mediated injury in a host with normal factor H structure and normal circulating concentrations suggests a limited affinity of factor H for the target tissue. It is noteworthy that the CAP has been implicated in models of several types of renal disease, including renal I/R, aHUS, dense deposit disease, focal segmental glomerulosclerosis, anti-neutrophil cytoplasmic antibody-associated vasculitis, and lupus nephritis.[4,10,14,22,23] The predilection of the kidney to be a primary site of injury may be a consequence of the particular repertoire of surface complement regulatory proteins expressed by the different renal cell types, and it may also be a consequence of the affinity of factor H for the various structures within the kidney. Complement activation on ischemic tubules, for example, may be the result of inadequate complement inhibition by factor H in the setting of decreased expression of Crry by the TECs.[13]

The concentration of complement proteins in interstitial fluid, vascular permeability, and local production of complement proteins may also be important determinants of complement activation in the renal tubulointerstitium. These factors likely contribute to the local balance of activation. It is possible, for example, that circulating factor H has only limited access to the TEC cell surface. In that case, *in vitro* assays, such as those shown in Figure 4, would not accurately represent the balance of complement proteins (activators and inhibitors) present on the TEC surface *in vivo*. The optimal design of tissue-targeted therapeutics might, therefore, have to consider tissue penetration of a molecule as equally important to target affinity. Local production of C3 by the tubules has been identified as an important factor in tubulointerstitial complement activation after renal I/R.[24] The absence of tubular C3 deposition in the $fH^{-/-}$ mice subjected to I/R, however, suggests that intact circulating C3 (which is reduced in the $fH^{-/-}$ mice) may be more important than locally produced C3 in our model.

Targeted inhibitors such as CR2-fH offer great promise as therapeutics. Because they specifically bind at sites of inflammation, they may offer favorable pharmacokinetics and may also cause less systemic immunosuppression than untargeted agents.[21] A therapeutic such as CR2-fH may be particularly useful for patients carrying factor H mutations, or for patients with pathologic CAP activation in tissues for which circulating factor H has limited affinity. It is notable

that the administration of CR2-fH considerably reduced the degree of CAP activation in the tubulointerstitium, even in the presence of circulating factor H. It is also possible that the administration of additional purified factor H would have been equally effective, although the concentration of factor H needed to achieve inhibition on TECs (roughly 1 mg/ml in undiluted serum) would require a considerable amount of protein.

In conclusion, these studies demonstrate that endogenous factor H plays an important role in limiting CAP activation on the TEC surface after renal I/R. However, CAP activation still causes tissue injury in the ischemic kidney, likely because of insufficient affinity or access of circulating factor H to this tissue surface. Therapeutic complement inhibitors should be effective for the treatment of diseases in patients with mutations in the complement regulatory proteins. These agents should also be beneficial in diseases, such as ischemic acute kidney injury, in which the body's normal complement regulatory mechanisms do not adequately control activation of the CAP. The expression of cell surface complement regulatory proteins and the affinity of factor H to the cell surface may be unique to each tissue. The common involvement of the CAP in many different types of renal disease indicates that complement regulation within the kidney is easily disrupted or overwhelmed, even in the absence of mutations in the complement regulatory proteins. A greater understanding of the function and limitations of the endogenous regulatory proteins within specific tissue sites may permit the development of more effective complement inhibitors, possibly even the development of tissue-specific therapeutic agents.

MATERIALS AND METHODS

Reagents

Antibodies used in these experiments include a polyclonal goat antibody for human factor H that crossreacts with mouse factor H (Quidel Corporation, San Diego, CA), a fluorescein isothiocyanate-conjugated polyclonal goat antibody to mouse C3 (MP Biomedicals, Solon, OH), and a monoclonal antibody (mAb 7E9) to a non-C3d-binding region of mouse CR2.Species-appropriate secondary antibodies from Jackson ImmunoResearch (West Grove, PA) were used where necessary, and fluorescein isothiocyanate-streptavidin was used to detect biotinylated proteins in tissues. To purify mouse factor H, serum from wild-type mice was buffer exchanged with 35 mmol/l NaCl and loaded onto a HiTrap heparin HP column (GE Amersham, Piscataway, NJ). Bound proteins were eluted using a linear gradient of NaCl and were then separated by size using a Sephadex 26/60 Superdex 200 column (GE Amersham). The purity and identity of the isolated protein was verified by Coomassie staining and by western blot analysis. A recombinant fragment of mouse factor H encompassing the 19th and 20th short consensus repeat of the protein was generated in a fashion similar to that previously described for the equivalent fragment of the human protein (see Supplementary Materials and Methods online and Ferreira *et al.*[16]).

Protocol for induction of renal I/R

Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice with targeted deletion of the gene for factor H ($fH^{-/-}$ mice) were generated as previously described,[14] and were backcrossed >10 generations onto a C57BL/6 background. Mice, 8 to 10 weeks old and weighing 20–25 g, were anesthetized with 60 mg/kg ketamine plus 10 mg/kg xylazine (both from Vedco, St Joseph, MO) injected intraperitoneally. Mice were placed on a heating pad to maintain their body temperature during surgery. Laparotomies were then performed and the renal pedicles were located and isolated by blunt dissection as previously described.[10] The pedicles were clamped with surgical clips (Miltex Instrument Company, York, PA), and occlusion of blood flow was confirmed by visual inspection of the kidneys. The clamps were left in place for 24 min and then released. The kidneys were observed for approximately 1 min to ensure blood re-flow, then fascia and skin were sutured with 4–0 silk (United States Surgical, Norwalk, CT). Sham surgery was performed in an identical fashion, except that the renal pedicles were not clamped. The mice were volume resuscitated with 0.5 ml of normal saline and kept in an incubator at 29 °C to maintain body temperature. After 24–72 h, the mice were anesthetized, and blood was obtained by cardiac puncture. Laparotomy was performed and the kidneys were harvested. All animal procedures were in adherence to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Western blot analysis

Either 100 μg of protein or 1 μl of serum was resolved by electrophoresis with a 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. Factor H was detected in the lysates using a polyclonal goat anti-human antibody (Quidel) diluted 1/100 or a monoclonal antibody to mouse factor H (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/500. Serum C3 was detected using horseradish peroxidase-conjugated polyclonal goat antibody to mouse C3 (MP Biomedicals). Appropriate secondary antibodies from Jackson Immunoresearch Laboratories were used.

Enzyme-linked immunosorbent assays

Plasma C3a levels were measured by enzyme-linked immunosorbent assay using monoclonal antibodies and standards from BD Biosciences (Rockville, MD) according to the manufacturer's instructions. Serum levels of biotinylated rH19-20 were measured by an enzyme-linked immunosorbent assay-type assay. Briefly, serum samples were diluted 1:50 and incubated with streptavidin-coated plates (Thermo Scientific, Rockville, IL). The bound rH19-20 was then detected using the antibody to factor H.

SUN and creatinine measurements

SUN and creatinine were measured using an Alfa Wasserman ACE Chemistry Analyzer (West Caldwell, NJ).

Statistics

Multiple group comparisons were performed using analysis of variance with post-test according to Tukey. Comparison between two groups was performed with a two-tailed Student's *t*-test. A *P*-value of <0.05 was considered statistically significant. Results are reported as mean ± s.e.

DISCLOSURE

JMT is a stockholder in and consultant for Taligen Therapeutics. VMH is a stockholder and is the chief scientific officer for Taligen Therapeutics. MKP is an officer of and has a financial interest in Complement Technology (www.ComplementTech.com), a supplier of

complement reagents. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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